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(54) Title: METHODS INVOLVING A T CELL PROTEIN TYROSINE PHOSPHATASE

(57) Abstract: This invention relates to T cell protein tyrosine phosphatase (TCPTP) and more particularly to its role in cell signaling and interaction with the JAK family of tyrosine kinases. In particular, the invention involves the use of TCPTP for the development of treatments for malignancies and autoimmune conditions involving inappropriate JAK kinase signaling as well as for the identification of inhibitors and activators of this phosphatase.

METHODS INVOLVING A T CELL PROTEIN TYROSINE PHOSPHATASE

Field of the Invention

This invention relates to T cell protein tyrosine phosphatase (TCPTP) and more particularly to the ellucidation of the role of TCPTP in cell signaling.

Background of the Invention

Throughout this application, various references are cited in parentheses to describe more fully the state of the art to which this invention pertains. The disclosure of these references are hereby incorporated by reference into the present disclosure. For convenience, a list of the references is appended to the description.

Intracellular signaling mediated by the phosphorylation of tyrosyl residues of proteins is regulated by two opposing families of enzymes: protein tyrosine kinases and protein tyrosine phosphatases. The phosphorylation of tyrosine is a key process in the regulation of cell growth and metabolism either due to the intrinsic ability of phosphorylation to cause activation of protein function or by its ability to form a basis for protein scaffolding to occur.

Protein tyrosine phosphatases (PTPases) form a large superfamily of enzymes that counteract the action of tyrosine kinases in cellular systems by catalyzing the hydrolysis of phosphorylated tyrosyl residues. Classically, tyrosine kinases have been seen as the main players in signal transduction, while the phosphatases have been relegated to a housekeeping role. Increasing evidence from the literature suggests that the phosphatases in fact play a critical role in maintaining the dynamic nature of many signaling pathways. This counterplay between tyrosine kinases and phosphatases allows extracellular signals to regulate cellular physiology.

PTPases are characterized by the presence of a 240 amino acid catalytic domain containing the signature motif (I/V)HCXAGXXR(S/T). The

cysteine in this motif is essential for the catalysis of phosphate ester hydrolysis. The current number of PTPases in the human genome is unknown, but is estimated at somewhere between 100 to 500, with about 48 full length sequences currently known (Tonks et al., 1996; Li et al., 2000). This number makes the PTPase superfamily one of the largest in the mammalian genome. PTPases can be further divided into those enzymes that specifically recognize only phosphotyrosine and those enzymes, known as the dual-specificity phosphatases, that recognize phosphotyrosine as well as phosphoserine and phosphothreonine residues. A subclass of phosphatases has been found which also hydrolyze phospholipid substrates.

PTPases can be further divided into transmembrane and intracellular members of this family. In addition, a number of PTPases contain other protein interaction motifs such as proline rich sequences, SH2 domains, PDZ domains, and tyrosine phosphorylation sites themselves. PTPase function and substrate specificity seem to be regulated by a variety of interacting proteins as well as by post translation modifications.

The T cell protein tyrosine phosphatase (TCPTP) was first identified in humans as two splice isoforms of the same gene, a 45 kDa isoform named TCPTPα and a 48kDa isoform named TCPTPβ. Northern blot analysis has shown that the transcript is ubiquitously expressed, but in substantially higher amounts in hematopoietic tissues. TCPTPα has a relatively simple structure with an N terminal PTPase domain and a C terminal nuclear localization signal. Both isoforms are identical except for the 3' end of the mRNA, which in TCPTPβ encodes a hydrophobic stretch of amino acids at the C terminus which results in retention of TCPTPβ in the endoplasmic reticulum. The TCPTPβ isoform is found in substantially lower amounts in the cell than the TCPTPα isoform (Ibarra-Sanchez et al., 2000).

To elucidate the role of a phosphatase in intracellular signaling, it is essential to identify its putative substrates. The Applicants have now

identified the JAK kinase family of tyrosine kinases as physiological substrates of TCPTP.

Summary of the Invention

It is now demonstrated that TCPTP plays a direct role in the control of tyrosine phosphorylation mediated cell activation in hematopoietic cells and tissues. Using a substrate trapping approach (Tiganis et al., 1998) TCPTP was demonstrated to target and thus regulate JAK activity. TCPTP is identified as a central negative regulator of cytokine signaling via a TCPTP-JAK interaction in hematopoietic cells. As such, TCPTP can now be used in a variety of methods in the regulation of immune homeostasis.

The invention provides the identification of inhibitors and activators of TCPTP which can be used for regulating JAK signaling. In one aspect, TCPTP inhibitors can be identified that can be used for the decrease of TCPTP activity leading to increased JAK kinase activity in those conditions where such TCPTP inhibition is desirable. In another aspect, TCPTP activators can be identified and be used to increase TCPTP activity leading to decreased JAK kinase activity in conditions where such TCPTP activation is desirable. This also allows for the development of pharmaceuticals that can be used to treat a variety of clinical disorders that involve inappropriate JAK activation. Such disorders may include but are not limited to hematologic malignancies, general defects in lymphocyte proliferation and hematopoiesis, autoimmune diseases and inflammation.

The present invention identifies T cell protein tyrosine phosphatase (TCPTP) is involved in the T cell signaling pathway downstream of the IL-2 receptor. The invention also identifies previously unrecognised substrates on which TCPTP acts, Janus kinase 1 (JAK1) and Janus kinase 3 (JAK3).

TCPTP's role in signaling downstream of the IL-2 receptor helps to understand why knock out of the TCPTP gene leads to the appearance of hematopoietic defects.

The invention in one aspect, is directed to the functional interaction of TCPTP with JAK tyrosine kinases.

The invention in further aspects provides methods for the dephosphorylation and inactivation of JAK tyrosine kinases, the method comprising contacting a TCPTP or a TCPTP analog, functional derivative or biologically active fragment thereof with a substrate JAK tyrosine kinase.

In accordance with an aspect of the invention is a method of diagnosing an immune disorder comprising determining the presence of a defect in TCPTP/JAK kinase interaction. A defect can be a decrease in the binding of TCPTP to JAK kinase leading to increased tyrosine phosphorylation indicative of an immune disorder. The defect may also be represented by a decrease in the dephosphorylation of JAK kinase by TCPTP.

According to another aspect of the present invention is a method of screening TCPTP analogs for their ability to bind to JAK kinase comprising:

i) providing a TCPTP analog;

ii) incubating said analog in the presence of a JAK kinase; and

iii) comparing the level of binding of said analog with a TCPTP control.

According to another aspect of the invention is method of screening TCPTP analogs for their ability to bind to JAK kinase without causing dephosphorylation of the JAK kinase comprising:

i) providing an analog that binds to a JAK kinase;

ii) incubating said analog in the presence of JAK kinase;

iii) determining the level of JAK kinase dephosphorylation; and

iv) selecting an analog that lacks JAK dephosphorylation activity.

The invention in another aspect, provides methods for the regulation of immune responses involving JAK tyrosine kinase activation. Such methods may include the use of TCPTP protein, an analog thereof, a functional derivative thereof or functional fragment thereof to target and decrease hyperphosphorylation of a JAK kinase. Alternatively, a functioning TCPTP nucleic acid sequence can be used in the present invention. The human

TCPTP sequence is disclosed in Cool et al., 1990, Accession No. M25393. The mouse TCPTP sequence is disclosed in Mosinger et al., 1992, Accession No. M81477.

The present invention also relates to functionally equivalent variants of TCPTP protein. "Functionally equivalent variants" or "analogs" includes peptides with partial sequence homology, peptides having one or more specific conservative and/or non-conservative amino acid changes, peptide conjugates, chimeric proteins, fusion proteins and peptide encoding nucleic acids. The functionally equivalent variants maintain the biological activity of the native peptide. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. Preferred substitutions are ones which are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well know in the art and typically include substitutions within the following groups: glycine, alanine; valine; isoleucine; leucine; aspartic acid, glutamic acid; asparagine, glutamine;serine, threonine; lysine, arginine, tyrosine and phenylalanine.

The invention also may involve the identification and use of peptide mimetics of TCPTP protein or peptide sequence. The term "peptide mimetic" or "mimetic" is intended to refer to a substance which has the essential biological activity of the TCPTP polypeptide. A peptide mimetic may be a peptide-containing molecule that mimics elements of protein secondary structure (Johnson et al., 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen, enzyme and substrate or scaffolding proteins. A peptide mimetic is designed to permit molecular interactions similar to the natural molecule. A mimetic may not be a peptide at all, but it will retain the essential biological activity of natural TCPTP polypeptide.

In addition, the present invention provides methods of screening drugs (i.e. small molecule inhibitors and/or activators) for cancer therapy to identify suitable drugs for restoring or up-regulating TCPTP gene product function.

The present invention also provides the means necessary for production of gene-based therapies directed at cancer cells, especially hematopoietic cancerous cells. The therapeutic agents may take the form of polynucleotides comprising all or a portion of the TCPTP nucleic acid sequence placed in appropriate vectors or delivered to target cells in more direct ways such that the function of the TCPTP protein phosphatase activity is reconstituted. Therapeutic agents may also take the form of polypeptides based on either a portion of, or the entire protein sequence of TCPTP. These may functionally replace the activity of TCPTP *in vivo*. *Ex vivo*, therapies are also encompassed within the invention.

The invention enables a method for screening a candidate compound for its ability to affect the interaction of a T cell protein tyrosine phosphatase (TCPTP) with a member of the JAK family of kinases, comprising:

(a) providing a preparation containing a T cell protein tyrosine phosphatase, a JAK kinase and a candidate compound; and

(b) determining whether the candidate compound affects the interaction of the T cell protein tyrosine phosphatase with the JAK kinase.

The T cell protein tyrosine phosphatase may be obtained from mammalian hematopoietic cells, including T cells, or may be prepared by recombinant expression.

The JAK kinase may be obtained from hematopoietic cells or prepared by recombinant expression. JAK kinases 1 and 3 are preferred.

The effect of a compound on the interaction of TCPTP and a JAK kinase may be determined, for example, by looking at its effect on the binding of TCPTP and a JAK kinase. A suitable assay is conducted in which candidate compounds are tested for their ability to increase or decrease the binding of a JAK kinase and TCPTP. Suitable assays will be known to those

of skill in the art; for example, the binding assay described herein may be employed.

One of skill in the art is now enabled to screen tissue homogenates or cell lysates for proteins or other compounds which bind to a TCPTP protein. Once identified, such compounds may themselves provide new pharmaceutical agents or may serve as lead compounds for the development of further pharmaceutical agents. Methods of screening for binding partners of a novel protein are well known to those in the art and include techniques such as affinity chromatography, immunoprecipitation using antibodies specific for a TCPTP protein, the BIAcore system of Pharmacia and the yeast two-hybrid system.

The invention provides a method for identifying compounds which can modulate the expression of a TCPTP gene comprising;

- contacting a cell with a candidate compound wherein said cell includes a regulatory region of a TCPTP gene operably joined to a coding region; and

- detecting a change in expression of said coding region.

The invention further provides a method for identifying compounds which can selectively bind to a TCPTP protein comprising the steps of

- providing a preparation including at least one TCPTP protein or or portion thereof;

- contacting said preparation with a sample including at least one candidate compound; and

- detecting binding of said TCPTP protein or portion thereof to said candidate compound.

The invention further provides a method for identifying compounds which can modulate activity of a TCPTP protein comprising the steps of

- contacting a cell expressing a normal or mutant TCPTP protein with at least one candidate compound; and

- detecting a change in a marker of said activity.

In general, various assay screening methods may be used to identify any type of molecule that inhibits or activates TCPTP.

The invention further provides methods for reducing tyrosine kinase activity of hematopoietic cells/tissues, for reducing the expression of CD3ε on cell surfaces of hematopoietic cells or for downregulating the activity of the TCR complex.

Once identified by such a method, a candidate compound may be useful as a pharmaceutical or may serve as a lead compound in the design of further compounds which may be useful as pharmaceuticals.

Compounds which reduce TCPTP binding to a JAK kinase, and therefore will reduce TCPTP activity against JAK kinases, are potentially useful as pharmaceuticals for suppressing cytokine-stimulated hematopoietic cell proliferation, particularly IL2-stimulated proliferation. It appears that TCPTP by dephosphorylating a JAK kinase, turns off the JAK kinase. These compounds are therefore potentially useful to treat any disorder in which immune suppression is desirable, for example T cell and other hematopoietic cell malignancies, autoimmune disorders and in preparation for and after tissue or organ transplantation.

Compounds which increase TCPTP binding to a JAK kinase may increase TCPTP activity in relation to JAK kinases, and are potentially useful as pharmaceuticals to boost immune responses in any disorder in which cytokine-stimulated T cell or hematopoietic cell proliferation is deficient, for example, in patients with SCID or in cancer patients undergoing cancer treatments such as chemotherapy.

The invention further enables a method for screening a candidate compound for its ability to modulate the activity of T cell protein tyrosine phosphatase, comprising:

(a) providing an assay system in which an activity of T cell PTP may be measured;

(b) determining the effect of a candidate compound on the T cell PTP activity in the assay system.

As used herein, "activity" of TCPTP includes any functional activity as well as any downstream effect of any such functional activity.

For example, the effect of a candidate compound on IL2-stimulated proliferation *in vitro* of a T cell culture may be examined in an assay system such as that described in the examples herein.

The effect of a candidate compound on the ability of a TCPTP preparation to dephosphorylate a JAK kinase, preferably a JAK1 or JAK3 kinase may also be examined.

Additionally, the effect of a candidate compound on the activity of a JAK kinase, preferably a JAK 1 or JAK 3 kinase, may also be examined, for example by a method such as that described in Barber et al. (1994).

The identification of JAK1 and JAK3 as substrates for TCPTP permits the determination of the properties of a preferred compound to affect the interaction of TCPTP and JAK.

Recent evidence from the TCPTP knock out animals indicates that this phosphatase plays a critical role in regulating cellular proliferation in lymphocytes. This information, coupled with the identification of JAK 1 and 3 as substrates, lends further proof for TCPTP's role in proliferation. Deregulation of TCPTP control of proliferative signaling pathways could be an important factor in hematologic malignancies.

The knowledge of TCPTP's function allows for inhibitor design. The ability to develop inhibitors specific for TCPTP may not only be of use in chemotherapy, but also in immune suppression in surgical transplant operations.

When PTP1B, the closest family relative to TCPTP, is knocked out in mice, the animals are resistant to diet-induced obesity and diabetes and, more importantly, are completely viable. Potentially, inhibitors for PTP1B may reduce obesity and type 2 diabetes. The close identity of the TCPTP and PTP1B catalytic domains means, however, that potential small molecule inhibitors may inhibit both PTPases, with undesirable immune suppression due to TCPTP inhibition.

In accordance with a further embodiment of the invention, potential anti-diabetic and anti-obesity PTP1B inhibitors may now be screened for TCPTP inhibition to rule out any with the potential for immune suppression through TCPTP inhibition, and permit selection of those which selectively inhibit PTP1B without affecting TCPTP activity.

Conversely, compounds showing TCPTP inhibition and therefore potentially useful as immune suppressors, can be screened for inhibitory activity against PTP1B to rule out any unwanted effects on carbohydrate metabolism.

Description of the Figures

The present invention will be further understood from the following description with reference to the Figures, in which:

Figure 1 shows immunoblots illustrating the identification of potential substrates for TCPTP in T cells. 1(A) CTLL-2 cells were transfected with the indicated plasmids and subsequently stimulated with recombinant human IL-2 (100 U/mL) for the indicated time points. 1(A) TCPTP was immunoprecipitated and subjected to anti-phosphotyrosine immunoblotting to reveal putative substrates after SDS-PAGE and electrophoretic transfer. 1(B) A similar experiment was performed in DO 11.10 cells except cells were stimulated with anti-CD3E antibodies (10μg/ml) to cross-link the TCR.

Figure 2 shows immunoblots illustrating TCPTP binds JAK3, and not JAK1, after IL-2 stimulation. CTLL-2 cells were transfected with TCPTP-WT or DA, cytokine depleted, and stimulated with IL-2 for 15 mins. TCPTP immunoprecipitates were blotted with anti-phosphotyrosine antibodies to identify co-immunoprecipitating substrates. Membranes were stripped and reprobed with antibodies to both JAK1 and JAK3. All experiments were blotted for TCPTP to ensure equal immunoprecipitation of the PTP.

Figure 3 shows immunoblots illustrating that members of the JAK family are substrates for TCPTP. (A) COS7 cells co-transfected with TCPTP-DA the indicated JAKs and co-immunopreciptiation revealed by anti-phosphotyrosine and specific JAK immunoblotting. (B) TCPTP dephosphorylates both JAK2 and JAK3 *in vivo*. Co-transfection in COS7 cells followed by immunoprecipitation and anti-phosphotyrosine immunoblotting shows the effect of TCPTP-WT or DA on JAK phosphorylation.

Figure 4 shows immunoblots illustrating the interaction of TCPTP and the JAKs is specific for the PTP domain of TCPTP and the activation loop of the kinase. (A) CTLL-2 cells were transfected with DA trapping mutants for TCPTP, PTP-PEST, and PTP1B. IL-2 stimulated cells were processed for immunoprecipitation of the PTPs and putative substrates were revealed by SDS-PAGE and anti-phosphotyrosine immunoblotting. (B) CTLL-2 cells transfected with TCPTP-DA and stimulated with IL-2 in the presence or absence of sodium orthovanadate, a potent competitive inhibitor of PTPs. (C) The activation loop of the JAKs is the molecular target of TCPTP. JAK2 and either TCPTP-WT or DA was co-transfected and lysates were incubated with a pYIOO7/pYIOO8 phosphospecific JAK2 antibody (Biosource). The bottom panel shows that both TCPTP-WT and DA were equally expressed in lysates. (D) Primary amino acid sequence comparison of the JAKs with a putative PTP1B/TCPTP substrate recognition motif.

Figure 5 illustrates that TCPTP resides in both the cytoplasm and nucleus of T cells. CTLL-2 cells were starved or stimulated with IL-2 and fractionated in to cytosolic and nuclear compartments. Lysates from each fraction were blotted for TCPTP, and subsequently PARP to ensure the integrity of the nuclear fraction.

Figure 6 illustrates TCPTP regulates signaling events downstream of the IL-2 receptor.(A) Co-transfection of Myc-STAT5a and TCPTP-WT or DA in

CTLL-2 cells, followed by IL-2 stimulation and immunopreciptiation of the Myc-tagged STAT5a was performed to assess the effect of TCPTP on IL-2 induced STAT5 activation with phosphospecific antibodies. (B) Peripheral T cells from lymph nodes were isolated from wild-type (WT) and TCPTP knock out mice (KU) and stimulated with 1000 U/mL IL-2 for 15 minutes. Cell lysates were resolved by SDS-PAGE and immunoblotting was performed with activation specific STATS antibodies. The membrane was reprobed for STAT5 to assess loading of the STAT5 protein and with antibodies to TCPTP to verify the genotype of the cells used in the experiment. (C) Graphical representation of STATS tyrosine phosphorylation obtained from IL-2 stimulation of T cells from wild-type *(+/+)* or TCPTP deficient mice *(-/-)* quantified by densitometry. Shown is the mean of three independent experiments ± standard deviation. Data was analyzed by an unpaired, two-tailed, Student's T test comparing the results of the wild-type, set to 100, and the knock out (n = 3, $p < 0.05$).

Figure 7 illustrates JAK1 is hyperphosphorylated in macrophages from TCPTP gene targeted mice. (A) BMDMs from wild-type (WT) and TCPTP null mice (KU) were cultured and stimulated with 1000 U/mL IFN-γ for 15 minutes. Cells were lysed and JAK1 and JAK2 were immunoprecipitated and subjected to antiphosphotyrosine immunoblotting. (B) Lysates from this experiment were immunoblotted for TCPTP to ensure to genotype of the cells used.

Figure 8 illustrates hyperactivation of STAT1 from TCPTP deficient thymocytes. (A). Thymocytes from wild-type (WT) and TCPTP deficient mice (KU) were stimulated with 1000 U/mL IFN-α and IFN-γ for 15 minutes. Activation of STAT1 was assessed with a phosphotyrosine-specific STAT1 antibody. (B) Genotype of the cells used was confirmed with anti-TCPTP immunoblotting. Shown are the results of two independent and representative experiments.

Detailed Description of the Preferred Embodiments

The Applicant has identified the T-cell protein tyrosine phosphatase (TCPTP) as a central negative regulator of cytokine signaling. Members of the Janus family of tyrosine kinases (JAKs) are identified as the predominant physiological substrates of TCPTP. Inherent substrate specificity in the TCPTP-JAK interaction is demonstrated by the inability of other closely related PTP family members to form an *in vivo* interaction with the JAKs in hematopoietic cells. In keeping with a negative regulatory role for TCPTP in cytokine signaling, hyperphosphorylation of JAK1 in bone marrow derived macrophages (BMDMs), as well as hyperphosphorylation of the downstream effectors, STAT1 and STAT5, was observed in TCPTP deficient cells. TCPTP is shown *in vivo* via a catalytic domain with members of the JAK family of kinases, providing a novel mechanism for the regulation of cytokine receptor signaling.

The knowledge of the mechanism for cytokine receptor signaling in hematopoietic cells provides new uses for these phosphatases in the treatment of hematopoietic abnormalities, in particular, proliferative disorders of the hematopoietic system involving inappropriate tyrosine phosphorylation leading to increased and/or defective cell proliferation. Inhibitors of TCPTP may also be identified for conditions where inappropriate TCPTP activity is involved such as for example in immune suppression disorders. TCPTP may be used in such therapeutic methods or alternatively, mimetics or functional analogs and derivatives may be used. Both activators and inhibitors of TCPTP can be developed and used to directly alter cytokine mediated tyrosine phosphorylation leading to inappropriate cellular signaling and clinical disorders.

TCPTP acts on substrates downstream of cytokine receptors

Wild-type (WT) or D182A (DA) substrate trapping mutants were constructed of TCPTP to isolate potential substrates by co-immunoprecipitation *in vivo*. Because the phenotype of TCPTP deficient mice

was consistent with a defect in hematopoietic cell signaling, CTLL-2 cells were used, a cytotoxic T cell line dependent on IL-2 for growth, and DO 11.10 cells, a murine T cell hybridoma.. Cells were transfected with TCPTP-WT, TCPTP-DA, or empty vector, and stimulated with exogenous IL-2 or anti-CD3E antibodies, to activate the IL-2 receptor and the T-cell receptor (TCR), respectively. Putative substrates were revealed by immunoprecipitation of TCPTP and anti-phosphotyrosine immunoblotting, which detects proteins protected by the TCPTP-DA trapping mutant from dephosphorylation by endogenous PTPs.

In CTLL-2 cells stimulated with IL-2, two co-immunoprecipitating substrates were revealed by anti-phosphotyrosine immunoblotting (Figure 1A). Selective co-immunoprecipitation of these two substrates, from the numerous phosphoproteins present in the IL-2 stimulated lysates of these cells, indicates specificity in substrate recognition by TCPTP. In DO 11.10 cells stimulated with anti-CD3 antibodies, a faint, but inducibly co-immunoprecipitating protein was observed at 200 kDa (Figure 1B). The experiments focused on the identification of substrates downstream of the IL-2 receptor.

Identification of JAK3 as an *in vivo* substrate of TCPTP

Based upon the molecular weight of the co-immunoprecipitating bands and the observed inducible tyrosine phosphorylation of these proteins following IL-2 stimulation, it appeared that these putative substrates may be JAK1 and JAK3, both of which are activated after IL-2 receptor engagement. Re-probing of the blot from this experiment with anti-JAK1 and anti-JAK3 antibodies, revealed that only JAK3, and not JAK1, was inducibly co-immunoprecipitated in the substrate trapping experiment (Figure 2). The unknown substrate, termed pp 130, was not identified by Western blotting for a variety of known proteins including the E3 ubiquitin ligase c-CBL and the tyrosine kinase c-ABL. It has recently been reported that JAK3 is activated after TCR stimulation [14], but a phosphotyrosine containing protein

corresponding to the size of JAK3 was not co-immunoprecipitated in the experiments in DO 11.10 cells.

Members of the JAK family are substrates for TCPTP

It was investigated whether other JAK family members are substrates for TCPTP. Co-transfection of TCPTP-DA with JAK1, JAK2, JAK3, and TYK2 followed by immunoprecipitation of TCPTP and anti-phosphotyrosine blotting, indicated that TCPTP formed complexes with all the JAK family members (Figure 3A). These results were confirmed by blotting with specific anti-JAK antibodies. It was reasonable to assume that putative substrates of PTPs should be efficiently dephosphorylated *in vivo.* Co-transfection of JAK2 and JAK3 with TCPTP-WT resulted in substantial dephosphorylation of the kinases, while TCPTP-DA protected both JAKs from dephosphorylation (Figure 3B).

Specificity of the TCPTP-JAK interaction

To determine whether the TCPTP-JAK3 interaction is specific for TCPTP and not other PTP family members, CTLL-2 cells were transfected with DA substrate trapping mutants of PTP-PEST, a member of the PEST family of PTPs known to play a role downstream of antigen receptors [15], and PTP-1B, a PTP that shares 74% sequence identity with TCPTP within the catalytic domain, along with TCPTP. Only TCPTP formed a substrate trapping complex with JAK3 and ppl3O, while neither PTP-PEST nor PTP-1B bound these putative substrates *in vivo,* although PTP1B-DA did co immunoprecipitate with a distinct set of substrates (Figure 4A, and data not shown).

The phenotypes of PTP1B and TCPTP deficient mice differed strikingly [12,16,17]. PTP1B gene targeted mice are resistant to diet-induced obesity and diabetes, while TCPTP deficient mice display hematopoietic abnormalities as described above. Due to the high similarity within the respective catalytic domains of TCPTP and PTP1B they are predicted to

target similar substrates. The present experiments indicate that other factors are involved, that may include tissue specific expression, subcellular localization of these PTPs [18], and other higher order interactions that could generate the observed differences in substrate specificity.

Mapping the TCPTP-JAK interaction

To assess whether the interaction of TCPTP and JAK3 is mediated via the PTP domain, we performed an IL-2 stimulated co-immunoprecipitation from CTLL-2 transfectants in the presence or absence of sodium orthovanadate, a potent competitive inhibitor of PTPs. In the presence of this inhibitor, all binding of phosphotyrosine proteins to TCPTP-DA was abolished, implicating the PTP domain as the mediator of the TCPTP-JAK interaction (Figure 4B).

Recent structural and kinetic data, describing the catalytic domain interaction of PTP1B, a close relative of TCPTP, complexed to the insulin receptor kinase activation loop phosphotyrosines, has been reported [19]. Kinetic evidence supports the structural model that PTP1B prefers to bind tandem phosphotyrosine motifs within polypeptides present in a consensus sequence of D/E-pYpY-K/R. JAK family members contain a similar activation loop motif, and because of the high homology of the catalytic domains of TCPTP and PTP1B, these sites most likely represent the molecular target of TCPTP (Figure 4D). Using a site specific antibody directed against the activation loop phosphotyrosines, pYI007/pYI008, of JAK2, it was demonstrated that this was the site of dephosphorylation by TCPTP (Figure 4C). Dephosphorylation of these residues by TCPTP would be predicted to decrease JAK kinase activity, suggesting a negative regulatory function for TCPTP in cytokine signaling.

TCPTP is found in both the cytoplasm and nucleus of T cells

TCPTP contains a nuclear localization signal and can shuttle between the nuclear and cytoplasmic compartments as observed in interspecies

heterokaryon assays [1 8,20,21]. To determine if TCPTP could act on JAKs in the cytoplasm, CTLL-2 cells were fractionated, either cytokine depleted or stimulated with IL-2, in order to determine if a cytoplasmic pool of TCPTP existed. Approximately 10% to 20% of TCPTP was found to be cytoplasmic, and its localization seemed to be independent of the effects of IL-2 stimulation (Figure 5).

STAT5 phosphorylation downstream of the IL-2 receptor is regulated by TCPTP

Binding of cytokines to their receptors leads to the activation of JAKs and subsequent tyrosine phosphorylation of STAT polypeptides, required for their dimerization and translocation to the nucleus where they activate target genes [2]. The effect of both TCPTP over-expression and loss of function on the downstream effector STATS in the IL-2 signaling pathway was investigated. CTLL-2 cells were co-transfected with a Myc-tagged STAT5a construct and either TCPTP-WT, TCPTP-DA, or vector alone. The cells were depleted of cytokine and subsequently stimulated with IL-2. Anti-Myc immunoprecipitates were blotted using a phosphotyrosine specific STAT5 antibody, which indicates the level of STAT5 activation. In the presence of vector alone, activation of STAT5 proceeded normally in response to IL-2 (Figure 6A). When co-transfected with TCPTP-WT, STAT5 activation was impaired, as observed by the decrease in tyrosine phosphorylation, while co-transfection the TCPTP-DA mutant did not hinder the activation of STAT5. To verify these results using a loss of function approach, isolated primary lymph node derived T cells from either wild-type or TCPTP deficient mice were isolated, which were subsequently stimulated with IL-2. IL-2 stimulated T cells showed hyperphosphorylation of STAT5 from TCPTP null animals as compared to cells from wild-type controls (Figure 6B and 6C). These results are consistent with a negative regulatory function for TCPTP in cytokine signaling via dephosphorylation of JAK3 as demonstrated by the previous data.

Hyperphosphorylation of JAK1 is observed in TCPTP null macrophages

In order to determine whether JAK phosphorylation was increased in the TCPTP null mice, as well as investigating the role of TCPTP in additional cytokine signaling pathways, bone marrow derived macrophages (BMDMs) were cultured from both wild-type and knock out animals. BMDMs were stimulated with IFN-y, a potent activator of macrophages, and both JAK1 and JAK2 were immunoprecipitated from cell extracts. Anti-phosphotyrosine immunoblotting of JAK1 immunoprecipitates revealed that the loss of TCPTP resulted in a significant increase in tyrosine phosphorylation of this JAK family member (Figure 7A). Interestingly, JAK2 immunopreciptates blotted with anti-phosphotyrosine antibodies showed little or no effect on its activation after cytokine stimulation from TCPTP deficient BMDMs (Figure 7A). The genotype of the cells used in this experiment was verified by blotting for TCPTP in cell extracts (Figure 7B). This result not only substantiates that TCPTP is a negative regulator of JAK kinases *in vivo,* but also illustrates substrate specificity of TCPTP for distinct JAK family members within the context of different cytokine signaling pathways.

Increased activation of STAT1 in TCPTP deficient cells

In accordance with the hyperphosphorylation of JAK1 observed in BMDMs from TCPTP deficient animals, the consequences of this effect on STAT 1 activation after IFN stimulation was investigated. In primary thymocytes, from both wild-type and TCPTP gene targeted animals stimulated with IFN-y, blotting with a phosphotyrosine specific STAT1 antibody revealed an increase in STAT1 activation in TCPTP deficient thymocytes (Figure 8A). Interestingly, thymocytes from wild-type and TCPTP null animals were also stimulated with IFN-α, a cytokine that activates both JAK1 and TYK2, and an increase in STAT 1 tyrosine phosphorylation was also observed in the TCPTP deficient cells (Figure 8B). In both experiments the genotype of the cells used was confirmed by blotting for TCPTP (Figure 8A and 8B). These results

indicate that TCPTP negatively regulates a variety of cytokine signaling pathways *in vivo*.

The molecular events that negatively regulate cytokine signaling pathways remain largely unknown. The SOCS and PIAS family of proteins, as well as the PTPs SHP-1 and CD45, have been reported to play a role in regulating cytokine signaling events [22]. SHP-1 deficient mice display gross hematopoietic abnormalities such as autoimmunity and macrophage hyperactivation that seem to indicate a negative role in cytokine signaling [23]. SHP-I is known to play a well characterized role in the negative regulation of antigen receptor signaling, as both T and B cells from SHP-1 deficient animals are hyperproliferative *in* vivo [24]. In SHP-1 deficient mice, hyperphosphorylation of JAK1 is observed in interferon signaling [25], and prolonged phosphorylation of JAK2 is observed after injection of growth hormone[26]. In the context of erythropoietin receptor signaling, recruitment of SHP- 1 to the tyrosine phosphorylated receptor chains enables its attenuation of JAK2 activation [27]. A direct catalytic domain interaction of SHP-1 with the JAKs has not been demonstrated, thereby leaving some uncertainty as to the molecular mechanism of SHP- 1 negative regulation of cytokine signaling.

The transmembrane PTP CD45 has recently been implicated in cytokine signaling and as a regulator of the JAK family [28]. Mice homozygous for a deletion in exon 6 of the CD45 gene have normal development of B cells, but thymocyte development is halted at the CD4+CD8+ double positive stage [29], although in a separate report certain sub-populations of B cells in CD45 null mice have been shown to be affected [30]. Antigen receptor driven signals are defective in both T and B cells, as well as in IgE promoted degranulation of mast cells [29,30]. While cells from CD45 mutant mice show hyperactivation of all JAKs, a functional association of the PTP domain with the putative substrates *in vivo* was not demonstrated [28]. *CD45* may play a discrete role in cytokine signaling, but the phenotype of CD45 deficient animals suggests that antigen receptor signaling is most sensitive to the loss

of this PTP and other PTPs are present to regulate cytokine signaling.

The present invention now demonstrates a functional interaction of the catalytic domain of TCPTP with members of the JAK family of tyrosine kinases. Not only is JAK phosphorylation increased in TCPTP deficient BMDMs, but this also translates into increased STAT activation downstream of a variety of cytokine receptors. TCPTP may target certain members of the JAK family depending on the cytokine signaling pathway in question. A preferential interaction of TCPTP trapping mutants with JAK3 and not with JAK1 downstream of the IL-2 receptor is demonstrated. Similarly, BMDMs from TCPTP deficient mice show an increase in JAK1 phosphorylation after IFN-γ stimulation, while JAK2 phosphorylation may only be mildly affected.

These findings provide insight into biochemical basis for the phenotype observed in the TCPTP gene targeted mice. T cell secretion of IFN-γ during the immune response is known to play an important role in macrophage activation. Hypersensitivity of macrophages to IFN-γ would result in their hyperactivation and lead to the overproduction of inflammatory cytokines such as TNF-α and IL-I. Indeed what is observed in the spleen of TCPTP deficient mice is an increase number of activated macrophages, which is consistent with an acute inflammatory response [11].

Members of the JAK family are now demonstrated to be physiological substrates of TCPTP via a tandem *in vivo* substrate trapping approach and biochemical analysis of primary cells from TCPTP gene targeted mice. Through this combined strategy, a functional interaction of the catalytic domain of TCPTP with the activation loop tyrosines of the JAK tyrosine kinases is demonstrated, resulting in their dephosphorylation and inactivation, and observed hyperactivation of downstream cytokine signaling pathways in TCPTP deficient cells. These results indicate a fundamental role for TCPTP in cytokine signaling that correlates well with the observed phenotype of the knock out animals and describes a novel mechanism in the regulation of the immune response.

The knowledge of TCPTP being a negative regulator of tyrosine kinase activity, particularly JAK activity, provides a method for using TCPTP, or analogs and derivatives thereof having the same biological activity, for testing of compounds capable of enhancing or inhibiting the phosphatase activity. The ability of a compound under testing to modify phosphatase activity can be tested in an *in vitro* system wherein the test compound is added to a purified TCPTP or biologically active analogs and derivatives thereof, and the effects on biological enzyme activity measured using standard enzymological procedures well known to those of skill in art.

Alternatively, the action of a compound on TCPTP activity can be measured in a whole cell preparation using live or fixed cells, or a fraction derived from live or fixed cells. This method is useful for screening compounds acting on the protein, in particular, on the enzymatic biological activity of the protein. A test compound is incubated with cells, or with a preparation derived therefrom, which express high amounts of the TCPTP of this invention. The amount of cellular phosphotyrosine is measured, using methods well-known in the art (Honegger et al., Cell 51:199-209 (1987); Margolis et al., Cell 51:1101-1107 (1989)). The results are compared to results obtained in the absence of the test compound, or in the absence or presence of a known activator of TCPTP. In such studies, the action of the test compound in the presence of an activator tyrosine kinase can also be measured.

A compound which stimulates TCPTP activity will result in a net decrease in the amount of phosphotyrosine, whereas a compound which inhibits TCPTP activity will result in a net increase in the amount of phosphotyrosine.

Thus, the present invention provides methods of screening for drugs comprising contacting such an agent with a TCPTP polypeptide, derivative or fragment thereof and assaying (i) for the presence of a complex between the agent and the TCPTP polypeptide or fragment, or (ii) for the presence of a complex between the TCPTP polypeptide or fragment and a ligand, by

methods well known in the art. In such competitive binding assays the TCPTP polypeptide or fragment is typically labeled. Free TCPTP polypeptide or fragment is separated from that present in a protein:protein complex, and the amount of free (i.e., uncomplexed) label is a measure of the binding of the agent being tested to TCPTP or its interference with or promotion of TCPTP:ligand binding, respectively. One may also measure the amount of bound, rather than free, TCPTP. It is also possible to label the ligand rather than the TCPTP and to measure the amount of ligand binding to TCPTP in the presence and in the absence of the drug being tested. Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the TCPTP polypeptides and is described in detail in published PCT application WO 84/03564.

Briefly, a method of screening for a substance which modulates activity of a polypeptide may include contacting one or more test substances with the polypeptide in a suitable reaction medium, testing the activity of the treated polypeptide and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances. A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances.

Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g., in a yeast two-hybrid system (e.g., Bartel et al., 1993). This system may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide. Alternatively, the screen could be used to screen test substances for binding to a TCPTP specific binding partner, or to find mimetics of the TCPTP polypeptide.

Once a substance which modulates or affects polypeptide activity is identified, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e., manufacture or formulation, or

a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

In a further embodiment of the invention, there are now provided novel methods of treatment of diseases associated with TCPTP function or dysfunction.

The TCPTP gene (nucleic acid sequence) or fragment thereof, where applicable, may also be employed in gene therapy methods in order to increase the amount of the expression products of such genes in cancer cells. Such gene therapy is particularly appropriate for use in both cancerous and pre-cancerous cells, in which the level of TCPTP polypeptide is absent or diminished compared to normal cells. It may also be useful to increase the level of expression of a given TCPTP gene even in those tumor cells in which the mutant gene is expressed at a "normal" level, but the gene product is not fully functional.

Gene therapy would be carried out according to generally accepted methods, for example, as described by Friedman (1991) or Culver (1996). Cells from a patient's tumor would be first analyzed by the diagnostic methods described above, to ascertain the production of TCPTP polypeptide in the tumor cells. A virus or plasmid vector, containing a copy of the TCPTP gene linked to expression control elements is prepared. The vector may be capable of replicating inside the tumor cells. Suitable vectors are known, such as disclosed in U.S. Pat. No. 5,252,479, PCT published application WO 93/07282 and U.S. Pat. No. 5,691,198. The vector is then injected into the patient, either locally at the site of the tumor or systemically (in order to reach any tumor cells that may have metastasized to other sites). If the transfected gene is not permanently incorporated into the genome of each of the targeted tumor cells, the treatment may have to be repeated periodically.

Gene transfer systems known in the art may be useful in the practice of the gene therapy methods of the present invention. These include viral and nonviral transfer methods. A number of viruses have been used as gene transfer vectors or as the basis for preparing gene transfer vectors, including

papovaviruses (e.g., SV40, Madzak et al., 1992), adenovirus (Berkner, 1992; Berkner et al., 1988; Gorziglia and Kapikian, 1992; Quantin et al., 1992; Rosenfeld et al., 1992; Wilkinson et al., 1992; Stratford-Perricaudet et al., 1990; Schneider et al., 1998), vaccinia virus (Moss, 1992; Moss, 1996), adeno-associated virus (Muzyczka, 1992; Ohi et al., 1990; Russell and Hirata, 1998), herpesviruses including HSV and EBV (Margolskee, 1992; Johnson et al., 1992; Fink et al., 1992; Breakefield and Geller, 1987; Freese et al., 1990; Fink et al., 1996), lentiviruses (Naldini et al., 1996), Sindbis and Semliki Forest virus (Berglund et al., 1993), and retroviruses of avian (Bandyopadhyay and Temin, 1984; Petropoulos et al., 1992), murine (Miller, 1992; Miller et al., 1985; Sorge et al., 1984; Mann and Baltimore, 1985; Miller et al., 1988), and human (Shimada et al., 1991; Helseth et al., 1990; Page et al., 1990; Buchschacher and Panganiban, 1992) origin. Most human gene therapy protocols have been based on disabled murine retroviruses, although adenovirus and adeno-associated virus are also being used.

Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Graham and van der Eb, 1973; Pellicer et al., 1980); mechanical techniques, for example microinjection (Anderson et al., 1980; Gordon et al., 1980; Brinster et al., 1981; Costantini and Lacy, 1981); membrane fusion-mediated transfer via liposomes (Felgner et al., 1987; Wang and Huang, 1989; Kaneda et al, 1989; Stewart et al., 1992; Nabel et al., 1990; Lim et al., 1991); and direct DNA uptake and receptor-mediated DNA transfer (Wolff et al., 1990; Wu et al., 1991; Zenke et al., 1990; Wu et al., 1989; Wolff et al., 1991; Wagner et al., 1990; Wagner et al., 1991; Cotten et al., 1990; Curiel et al., 1991; Curiel et al., 1992). Viral-mediated gene transfer can be combined with direct *in vivo* gene transfer using liposome delivery, allowing one to direct the viral vectors to the tumor cells and not into the surrounding nondividing cells. Alternatively, a retroviral vector producer cell line can be injected into tumors (Culver et al., 1992). Injection of producer cells would then provide a continuous source of vector particles.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and degradation of the endosome before the coupled DNA is damaged. For other techniques for the delivery of adenovirus based vectors, see Schneider et al. (1998) and U.S. Pat. No. 5,691,198.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is nonspecific, localized *in vivo* uptake and expression have been reported in tumor deposits, for example, following direct in situ administration (Nabel, 1992).

Expression vectors in the context of gene therapy are meant to include those constructs containing sequences sufficient to express a polynucleotide that has been cloned therein. In viral expression vectors, the construct contains viral sequences sufficient to support packaging of the construct. If the polynucleotide encodes TCPTP, expression will produce TCPTP. If the polynucleotide encodes an antisense polynucleotide or a ribozyme, expression will produce the antisense polynucleotide or ribozyme. Thus in this context, expression does not require that a protein product be synthesized. In addition to the polynucleotide cloned into the expression vector, the vector also contains a promoter functional in eukaryotic cells. The cloned polynucleotide sequence is under control of this promoter. Suitable eukaryotic promoters include those described above. The expression vector may also include sequences, such as selectable markers and other sequences described herein.

Gene transfer techniques which target DNA directly to hematopoeitic tissue and cells is preferred. Receptor-mediated gene transfer, for example, is accomplished by the conjugation of DNA (usually in the form of covalently closed supercoiled plasmid) to a protein ligand via polylysine. Ligands are

chosen on the basis of the presence of the corresponding ligand receptors on the cell surface of the target cell/tissue type. These ligand-DNA conjugates can be injected directly into the blood if desired and are directed to the target tissue where receptor binding and internalization of the DNA-protein complex occurs. To overcome the problem of intracellular destruction of DNA, coinfection with adenovirus can be included to disrupt endosome function. Patients who carry a TCPTP susceptibility allele are treated with a gene delivery vehicle such that some or all of their precursor cells receive at least one additional copy of a functional normal TCPTP allele. In this step, the treated individuals have reduced risk of cancer to the extent that the effect of the susceptible allele has been countered by the presence of the normal allele.

The invention also relates to the use of identified antagonists or agonists of TCPTP in pharmaceutical compositions intended for treatment of diseases or conditions associated with abnormal expression of TCPTP. Alternatively, the pharmaceutical compositions may be used to treat a disease or condition associated with normal TCPTP but one or more deficiencies downstream in the signal transduction pathway or even a condition without any down stream deficiencies.

In yet a further embodiment of the invention, TCPTP polypeptides, antibodies, peptides and nucleic acids of the present invention can be formulated in pharmaceutical compositions, which are prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa.). The composition may contain the active agent or pharmaceutically acceptable salts of the active agent. These compositions may comprise, in addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g.,

intravenous, oral, intrathecal, epineural or parenteral. For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood brain barrier. See for example, WO 96/11698.

For parenteral administration, the compound may dissolved in a pharmaceutical carrier and administered as either a solution of a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

The active agent is preferably administered in an therapeutically effective amount. The actual amount administered, and the rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc., is within the responsibility of general practitioners or specialists, and typically takes account of the disorder to be treated, the condition of the

individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in Remington's Pharmaceutical Sciences.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands. Targeting may be desirable for a variety of reasons, e.g. if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cell, e.g. in a viral vector such as described above or in a cell based delivery system such as described in U.S. Pat. No. 5,550,050 and published PCT application Nos. WO 92/19195, WO 94/25503, WO 95/01203, WO 95/05452, WO 96/02286, WO 96/02646, WO 96/40871, WO 96/40959 and WO 97/12635. The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are more tissue specific to the target cells. The cell based delivery system is designed to be implanted in a patient's body at the desired target site and contains a coding sequence for the active agent. Alternatively, the agent could be administered in a precursor form for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. See for example, EP 425,731A and WO 90/07936.

The present invention also relates to a method for preventing or treating diseases or conditions involving the activation of TCPTP, the method comprising administering, to a patient in need thereof, an effective dosage of a TCPTP protein of the invention or an antibody of the invention or a molecule that stimulates or inhibits enzymatic activity of an TCPTP protein of the invention.

In the case of cytokine mediated tyrosine kinase activation, tyrosine phosphorylation is linked to cell growth and to oncogenic transformation. Activation of a TCPTP, leading to dephosphorylation would serve as a

counterregulatory mechanism to prevent or inhibit growth, and might serve as an endogenous regulatory mechanism against cancer. Thus, mutation or regulation of this enzyme system may promote susceptibility to cancer.

The insulin receptor is also a tyrosine kinase, and phosphorylation of tyrosine in cells bearing insulin receptors would be associated with normal physiological function associated with insulin. Three specific tyrosine residues in the intracellular portion of the insulin receptor are phosphorylated when insulin binds to the extracellular domain. At the same time, the insulin receptor becomes an active enzyme which can phosphorylate itself or other proteins at tyrosine residues. Phosphorylation of all three specific intracellular tyrosines of the insulin receptor appears to be required for full tyrosine kinase activity. The fully active insulin receptor transmits the signal into the cell (such as skeletal muscle, liver, etc.) by phosphorylating intracellular proteins, which are thereby activated and convey the messages further downstream via the insulin signal transduction pathway. Thus, the well-known physiologic effects of insulin result from a cascade of phosphorylation events.

Insulin signal transduction is controlled tightly by enzymes of the PTP class, which can dephosphorylate, and in the case of the insulin receptor, deactivate, tyrosine kinases. The existence of PTPs with activity towards the insulin receptor can easily be demonstrated. In this setting, then, activation of a PTP would counteract insulin effects, whereas inhibition of the PTP should mimic insulin effects. In fact, treatment of whole cells such as skeletal muscle or adipocytes with pervanadate, which inhibits PTPs, induces an almost full insulin response (Fantus, I. G. et al., Biochemistry 28:8864-8871 (1989); Leighton, B. et al., Biochem. J. 276:289-292 (1989)). Once the PTP which specifically acts on the insulin receptor is identified, it can be employed in a high throughput screening system and for rational drug design, to identify compounds which, like pervanadate, inhibit the phosphatase and mimic the action of insulin.

Over-activity, or inappropriate activation, of a PTP would be expected to inhibit or totally prevent the action of insulin on cells, leading to diabetes (of

an insulin-resistant variety). Thus, susceptibility to diabetes may be associated with PTP dysregulation, and may be diagnosed by measurement of PTP activity, including PTP-S31.

In summary, JAK kinases have been identified as substrates for TCPTP. As such the TCPTP protein, analogs and functional derivatives thereof, and agents which modulate (activate or inhibit) TCPTP enzymatic activity may be identified and used to treat or prevent the development of diseases involving inappropriate tyrosine phosphorylation such as cancer, particularly in hematopoietic cells and tissues and well as diabetes.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

EXAMPLES

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of synthetic chemistry, protein and peptide biochemistry, molecular biology, histology and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

Materials and Methods

Unless specified all materials were obtained from Sigma. RPMI, DMEM, and Fetal Bovine Serum (FBS) were from Wisent, recombinant murine IL-2 was from Roche, while recombinant murine IFN-a, murine IFN-y,

and recombinant human M-CSF were from Calbiochem. Antibody 4G1 0 against phosphotyrosine, anti-JAK1, anti-JAK2, and anti-JAK3 antisera, and anti-PARP antibodies were from Upstate Biotechnology. Anti-TCPTP antibodies 3E2 and 6F3 were purified from tissue culture supernatants of the respective hybridomas. Monoclonal antibodies anti-JAK1 and anti-TYK2 were from BD Transduction Laboratories. All PCR reactions were performed using the *Vent* DNA polymerase (New England Biolabs) with primers synthesized by the ACGT Corporation (Toronto, ON). Complete protease inhibitor tablets were from Roche.

Cell culture

DO 11.10 cells were routinely cultured in RPMI supplemented with 10% *(v/v)* FBS, *5* U/mL penicillin, *5* mg/mL streptomycin sulfate, and *55* μM 2-mercaptoethanol (Gibco BRL) at 37°C with *5%* CO_2. CTLL-2 cells were cultured in the same medium as above with the addition of 2 U/mL IL-2. COS7 cells were cultured in DMEM containing 10% (v/v) FBS, *5* U/mL penicillin, and *5* mg/mL streptomycin sulfate.

Plasmids and mutagenesis

All wild-type or mutant cDNAs were placed in the pEF-BOS expression vector (a kind gift Dr. Gary Koretzky, University of Pennsylvania) in the $^{5'}$ClaI/XbaI$^{3'}$ sites. C-terminal Myc tags were introduced by including the epitope tag sequence in the anti-sense primer for PCR. The TCPTP-D 1 82A mutant was constructed using PCR with the following mutagenic primers (mutation underlined): (+) $^{5'}$tgaaacgagaaccatatctcac$^{3'}$, (-) $^{5'}$ctggaaccccaaa~g.ctg gc c$^{3'}$ and the following terminal primers: (+)$^{5'}$taaatcgatccaccatgtcggcaaccatcg$^{3'}$, (-) $^{5'}$gtgtctagattaggtgtctgtcaatcttgg$^{3'}$. The same terminal primers were used for pEF-TCPTP-WT. The PTP-PEST-WT cDNA was subcloned in to the pEF-BOS vector at the $^{5'}$ClaI/XbaI$^{3'}$ sites. The PTP-PEST-D199A mutant was created with the following primers: (+) $^{5'}$gaatcgattggaggatggagcaagtggag$^{3'}$, (+) $^{5'}$gtgaactggccagç.çcatgatg$^{3'}$, (-)

5'gtggtacctgtcactgtcctg3', and (-) 5'catcatggg~tggccagttcac3'. The resulting PCR product was cloned into the PTPPEST cDNA using a 5'ClaI site and an internal HindIII site. The PTP-1B WT and D181A cDNAs were amplified with the following primers: (+) 5'gaggatccgagatggaaaaggagttcg3' and (-) 5'gagcggccgcctatgtgttgctgttgaacag3', and subcloned in to the pEBG vector at the 5'BamHh/NotI3' sites. Constructs expressing JAK1, JAK3, and TYK2 were prepared by blunt ending the *5'* ends of the cDNAs with the T4 DNA polymerase (Roche) then digesting with XbaI. These modified cDNAs were ligated in to a 5'ClaI-blunt ended/XbaI3' digested pEF-BOS vector. STAT5a was cloned into the pEF4/Myc-His vector at theco RI/Not I ~ sites with the following primers: (+) 5tagaattcccaccatggcgggctggattcag3' and (-) 5tagcggccgcggacagggagcttctagc3. The integrity of all constructs was verified by fully sequencing the cDNA (Hospital for Sick Children, DNA sequencing facility).

Western blotting

Protein complexes were resolved by 8% or 10% SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore). For phosphotyrosine, membranes were blocked 60 mm in 1% BSA in TBST. 4G10 was added at ljtg/mL in blocking buffer for 60 mm at room temperature. The membranes were washed for 10 mm, *5* mm, and *5* mm and the appropriate secondary was used for *45* mm in blocking buffer. After repeating the above washes, the membranes were visualized via enhanced chemiluminescence (Amersham), and exposed to film (Kodak). For other antibodies, the blocking buffer used was *5%* non-fat dried milk (Carnation) in TBST. Membranes were stripped in *62.5* Tris (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol, and washed extensively before use.

Transfection and immunoprecipitation

CTLL-2 cells, at log phase growth, were washed twice with RPMI and resuspended at 20 x 106 cells/400jtL RPMI in a 0.4 cm electroporation

cuvette (Bio Rad). Plasmid DNA was added at 40~ig and incubated with the cells for 10 mm at room temperature. Cells were pulsed at 960p~F and 250 V then chilled on ice for 10 mm. Cells were resuspended in 20 mL CTLL-2 culture medium and grown for 16-18 hours as above. Transfectants were then washed twice with PBS and depleted of cytokine for four hours. Transfectants were stimulated with 100 U/mL IL-2 for the indicated time points at 37°C. DOI 1.10 cells were transfected as above, except stimulations were preformed with soluble anti-CD3E (145-2C11, PharMingen). The cells were quickly pelleted and washed with ice cold PBS and lysed in HNMETG (50 mM Hepes(pH *7.5),* 150 mM NaC1, *1.5* mM $MgCl_2$, 1.0 mM EGTA, 1.0% Triton X-100, 10% glycerol), without the addition of orthovanadate, for 20 mm at 4°C with gentle rotation. Sodium orthovanadate was added to a final concentration of 2mM for all other experiements. Lysates were cleared for 10 mm at 4°C and 1 0,000g and protein quantitated by the method of Bradford (BioRad). 1mg of lysate in 1 mL was pre-cleared with either protein A or G-sepharose and normal rabbit or mouse IgG for 30 mm at 4°C, then incubated with the desired antibodies for 90 mins prior to the addition of protein A or G-sepharose. The immune complexes were washed four times with HNMETG and eluted into 2 x SDS sample buffer. SDS-PAGE, transfer, and Western blotting were as above. Transfection of COS7 cells was performed using the Lipofectamine reagent (Gibco BRL) as described by the manufacturer.

Cellular fractionation

The method used is similar to that of Andrews and Faller [3 1]. CTLL-2 cells, cytokine depleted and IL-2 stimulated, were washed twice with ice cold PBS and resuspended in hypotonic buffer (10 mM Hepes(pH 7.9), 1.5 mM $MgCl_2$, 10 mM KC1, with Complete protease inhibitors) for 10 mm on ice. The cells were lysed with a dounce homogenizer (type A pestle) until ~*95%* of cells were disrupted as determined by Trypan Blue uptake. Nuclei and cellular debris were pelleted for 10 sec in a benchtop centrifuge and washed once in hypotonic buffer. Nuclei were extracted with high salt buffer (20 mM Hepes

(pH 7.5), *25%* glycerol, 420 mM NaC1, 1.5 mM $MgC1_2$, 0.2 mM EDTA, with Complete protease inhibitors) for 20 mm on ice. The extract was cleared by centrifugation at 10,000g at 4°C for *5* mm. 20~~tg of each fraction, along with 20~tg HNMETG lysate was resolved by SDS-PAGE. Immunoblotting was performed with 6F3 anti-TCPTP and anti-PARP to determine the validity of the fractionation procedure.

Isolation of primary cells

Primary thymocytes or lymph nodes were obtained from wild-type or TCPTP deficient mice at days 14 to 18 after birth. The thymus or lymph nodes were removed and placed in RPMI supplemented with 10% (v/v) FBS, *5* U/mL penicillin, *5* mg/mL streptomycin sulfate, and *55* μM 2-mercaptoethanol (Gibco BRL). Cell suspensions were prepared by passing through a 70 μi nylon mesh. Primary cells were rested at 37°C for 4 hours prior to stimulation. Lymph node derived T cells were stimulated with 1000 U/mL of IL-2 and thymocytes were stimulated with 1000 U/mL IFN-α or IFN-y for 15 minutes. Cells were washed once in ice cold Hanks balanced salt solution then lysed in HNMETG buffer containing 10mM NaF, 2mM Na_3VO_4, and 420mM NaCl to extract nuclear components. Lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphospecific STAT1 or STATS antibodies. Data was analyzed by densitometry with the wild-type value set to 100 in each experiment. Bone marrow derived macrophages were obtained by flushing the femurs of 14 day old wild-type or TCPTP deficient mice with DMEM containing 10% FBS and antibiotics. The cells were pelleted and resuspended in the above medium supplemented with 10 ng/mL M-CSF. Stromal cells and mature macrophages were removed by adherence to tissue culture plastic for 24 hours. The next day (day 1), non-adherent cells were resuspended at 1.5 to 2.0 x 10^5/mL of M-CSF containing medium, and plated in 100 mm tissue culture plates. At day 4, the medium was removed and replaced with fresh growth medium containing M-CSF. At day 7, cells were depleted of M-CSF for 16 to 18 hours and stimulated with 1000 U/mL IFN-y. Cells were lysed and

subjected to immunopreciptiation and Western blotting as described.

In vitro substrate trapping-

E. coli were transformed with the various pGEX constructs and grown for 16 hrs in LB medium with 100 μg of Ampcillin (LBA). The culture was diluted 1:2 with LBA containing 1 mM IPTG and grown for 2 hrs at 37°C. The bacteria were pelleted and resuspended in PBS with 1% Triton-X100 and lysed via sonication at 50% power for 3 x 10 sec. The lysate was cleared and the GST-fusion proteins were bound to glutathione-sepharose (Pharmacia) for 45 min, then washed three times in PBS with 0.1% Triton-X100. Approximately 2μg of fusion protein was used for each experiment as compared to BSA standards. CTLL-2 cells, at log phase, were washed two times with PBS, and starved of cytokine for four hours. Cells were collected and resuspended at 10 x 10^6 cells/mL in RPMI and stimulated with 100 U/mL of recombinant murine IL-2 for 10 min at 37°C. After stimulation, the cells were pelleted, washed once with ice cold PBS, and lysed in HNMETG lysis buffer (50 mM Hepes, pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 10 % (v/v) glycerol, 1% (v/v) Triton-X100, Complete protease inhibitors(Roche)) supplemented with 5 mM iodoacetic acid (IAA), for 20 min with gentle rotation at 4°C. Unreacted IAA was removed by incubating cell lysate with 10 mM DTT for 20 min at 4°C. The lysate was clarified by centrifugation at 10,000g for 10 min at 4°C. Protein concentration was determined via the Bradford Assay (Bio Rad), and 500μg of protein in 1 mL was pre-absorbed with 2 μg of GST bound glutathione-sepharose beads (GSH-beads). The cleared lysate was transferred to a tube containing 2μg GST, TCPTP-WT/CS/DA bound GSH-beads, and incubated for 90 min at 4°C. The beads were washed four times with HNMETG and eluted in 2 x SDS sample buffer. Protein complexes were resolved by 8% or 10% SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore).

For phosphotyrosine, membranes were blocked 60 min in 1% BSA in TBST. 4G10 was added at 1μg/mL in blocking buffer for 60 min at room temperature. The membranes were washed for 10 min, 5 min, 5 min and the appropriate secondary was used for 45 min in blocking buffer. After repeating the above washes, the membranes were visualized via enhanced chemiluminescence (Amersham), and exposed to film (Kodak). For other antibodies, the blocking buffer used was 5% non-fat dried milk (Carnation) in TBST. Membranes were stripped in 62.5 Tris (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol, and washed extensively before use.

In vitro GST fusion protein mixing assay

To identify the potential substrates complexed with the TCPTP DA, an *in vitro* GST fusion protein mixing assay was used. In this assay, the TCPTP wild-type or the CS or DA trapping mutants were immobilized to an affinity matrix, and either starved or IL-2 stimulated protein extracts were incubated with the recombinant proteins. As seen in Figure 3, top panel, two distinct tyrosine phosphorylated proteins formed complexes with the TCPTP DA fusion protein *in vitro*. Since it had been previously reported that the Janus kinases (JAK) 1 and 3 are rapidly tyrosine phosphorylated after IL-2 stimulation, it was attempted to identify these coprecipitating bands by immunoblotting with antibodies against JAK 1 and 3. As observed in figure 3, second panel from top, JAK 1 was coprecipitated inducibly upon IL-2 stimulation, while JAK3 was precipitated with the TCPTP DA matrix in both starved and stimulated cells (third panel from top). Coomassie staining of the membrane shows equal loading of the fusion proteins used in the assay (bottom panel).

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations

may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

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Claims

1. A method for regulating an immune response, the method comprising increasing the phosphatase activity of TCPTP in hematopoietic cells.

2. The method of claim 1, wherein said phosphatase activity of TCPTP is increased by upregulating expression of a TCPTP gene in said hematopoietic cells.

3. The method of claim 1, wherein said phosphatase activity is increased by delivering exogenous TCPTP to said hematopoietic cells.

4. The method of claim 3, wherein said exogenous TCPTP is selected from the group consisting of TCPTP protein, a functional analog of TCPTP, a biologically active fragment of TCPTP protein, a mimetic of TCPTP and a functional derivative of TCPTP protein.

5. The method of any one of claims 1 to 4, wherein increasing the phosphatase activity of TCPTP in said hematopoietic cells decreases tyrosine phosphorylation of JAK kinases in said cells.

6. A method of diagnosing an immune disorder comprising determining the presence of a defect in TCPTP - JAK kinase interaction.

7. A method according to claim 6, wherein said defect is manifested by a lower than normal binding of TCPTP to JAK kinase.

8. A method according to claim 6, wherein said defect is manifested by a lower than normal dephosphorylation of JAK kinase.

9. A composition for the treatment of a condition involving inappropriate JAK kinase activity, said composition comprising; (i) a molecule selected from the group consisting of;

a) a TCPTP protein;

b) a biologically active fragment of (a);

c) a mimetic of a); and

d) a functional analog of a), b) or c); and

(ii) a pharmaceutically acceptable carrier.

10. The composition of claim 9, wherein said condition is a hematologic malignancy.

11. A method for the treatment of a condition involving inappropriate JAK kinase activation, said method comprising administrating a therapeutically effective amount of;

a) a TCPTP protein;

b) a biologically active fragment of (a);

c) a mimetic of a); and

d) a functional analog of a), b) or c),

for a time effective to reduce JAK kinase activation.

12. The method of claim 11, wherein said condition is a hematologic malignancy.

13. A method of screening a TCPTP analog for its ability to bind to JAK kinase comprising:

i) preparing a TCPTP analog;

ii) incubating said analog in the presence of a JAK kinase; and

iii) comparing the level of binding of said analog to said JAK kinase with a TCPTP control.

14. . The method of claim 13, wherein said TCPTP analog is a recombinant molecule derived by deletion or site-directed mutagenesis.

15. The method of claim 13, wherein said analog is prepared synthetically.

16. The method of claim 6, wherein said analog is a TCPTP mimetic.

17. An analog selected by the method of any one of claims 13 to 16.

18. Use of an analog of claim 17, in the treatment of a disorder characterized by abnormal TCPTP- JAK kinase interaction.

19. The use of claim 18, wherein said disorder is a hematologic malignancy.

20. A method of screening TCPTP analogs for their ability to bind to JAK kinase without causing dephosphorylation of the JAK kinase comprising:

i) providing an analog of claim 17;

ii) incubating said analog in the presence of a JAK kinase;

iii) determining the level of JAK kinase dephosphorylation; and

iv) selecting an analog that lacks JAK dephosphorylation activity.

21. An analog selected according to the method of claim 20.

22. Use of an analog as defined in claim 21, as an immunosuppressor.

23. Use according to claim 21, in transplantation.

24. Use according to claim 21, in the treatment of autoimmunity.

25. Use according to claim 21, in the treatment of hematopoietic malignancies.

26. An immunosuppressor molecule comprising a molecule that blocks the activation of JAK kinase by TCPTP.

27. The immunosuppressor molecule of claim 26, comprising a TCPTP anti-sense nucleic acid.

28. . The immunosuppressor of claim 26, comprising a mutated TCPTP molecule that binds to JAK kinase without causing dephosphorylation.

29. An immunostimulator molecule comprising a TCPTP analog that binds and activates JAK kinase.

30. A method for screening a candidate compound for its ability to affect the interaction of a T cell protein tyrosine phosphatase (TCPTP) with a member of the JAK family of kinases, comprising:

(a) providing a preparation containing a T cell protein tyrosine phosphatase, a JAK kinase and a candidate compound; and

(b) determining whether the candidate compound affects the interaction of the T cell protein tyrosine phosphatase with the JAK kinase.

31. The method of claim 30, wherein said JAK kinase is selected from the group consisting of JAK-1 and JAK-3.

32. A method for providing a host with resistance to diabetes, said method comprising decreasing the level of TCPTP activity in said host.

33. The use of a TCPTP protein, functional fragment thereof, derivative thereof, mimetic or functional analog thereof in the preparation of a medicament for the treatment of an immunological condition.

34. The use of claim 33, wherein said immunological condition is a hematopoeitc malignancy.

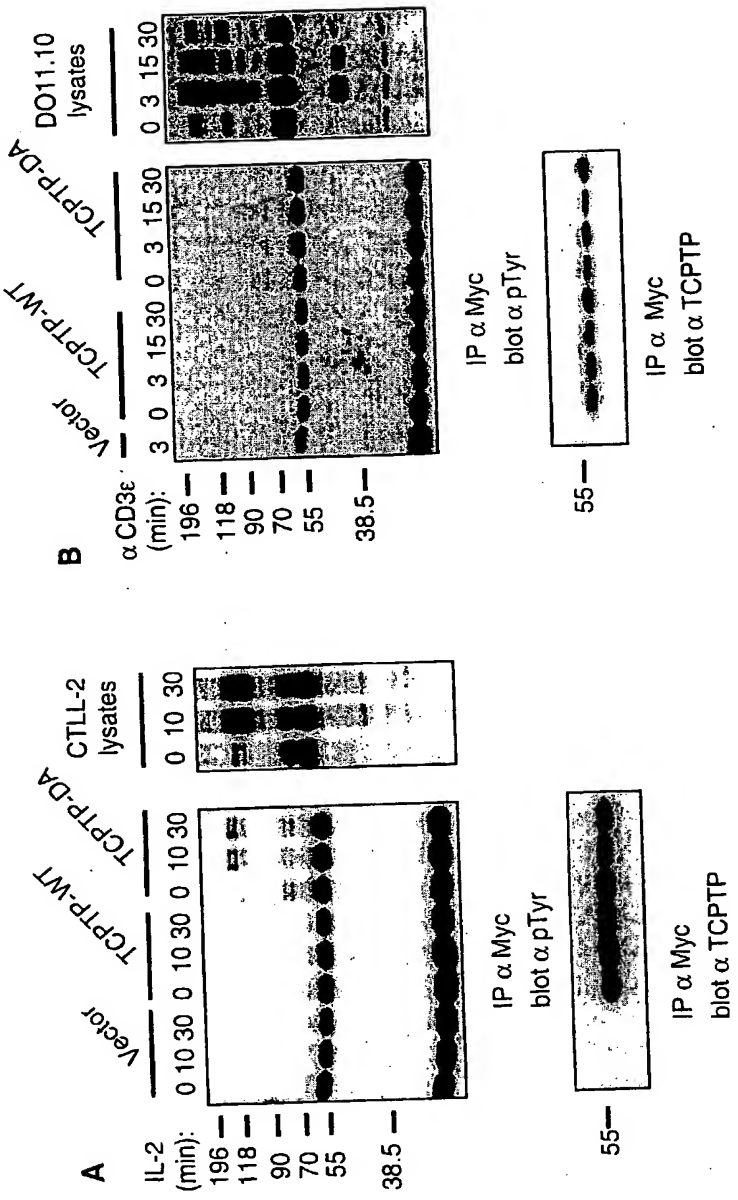
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Figure 1

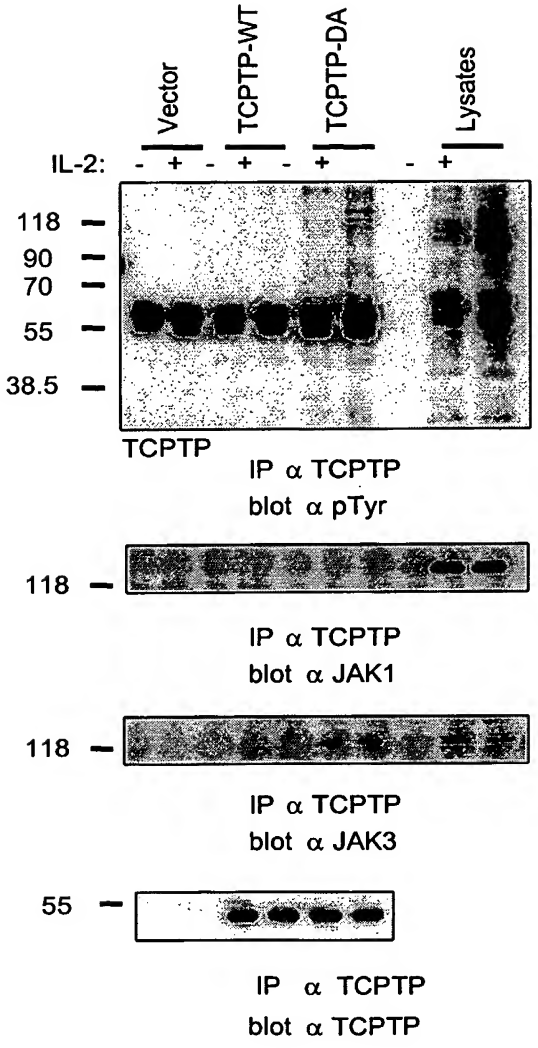
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Figure 2

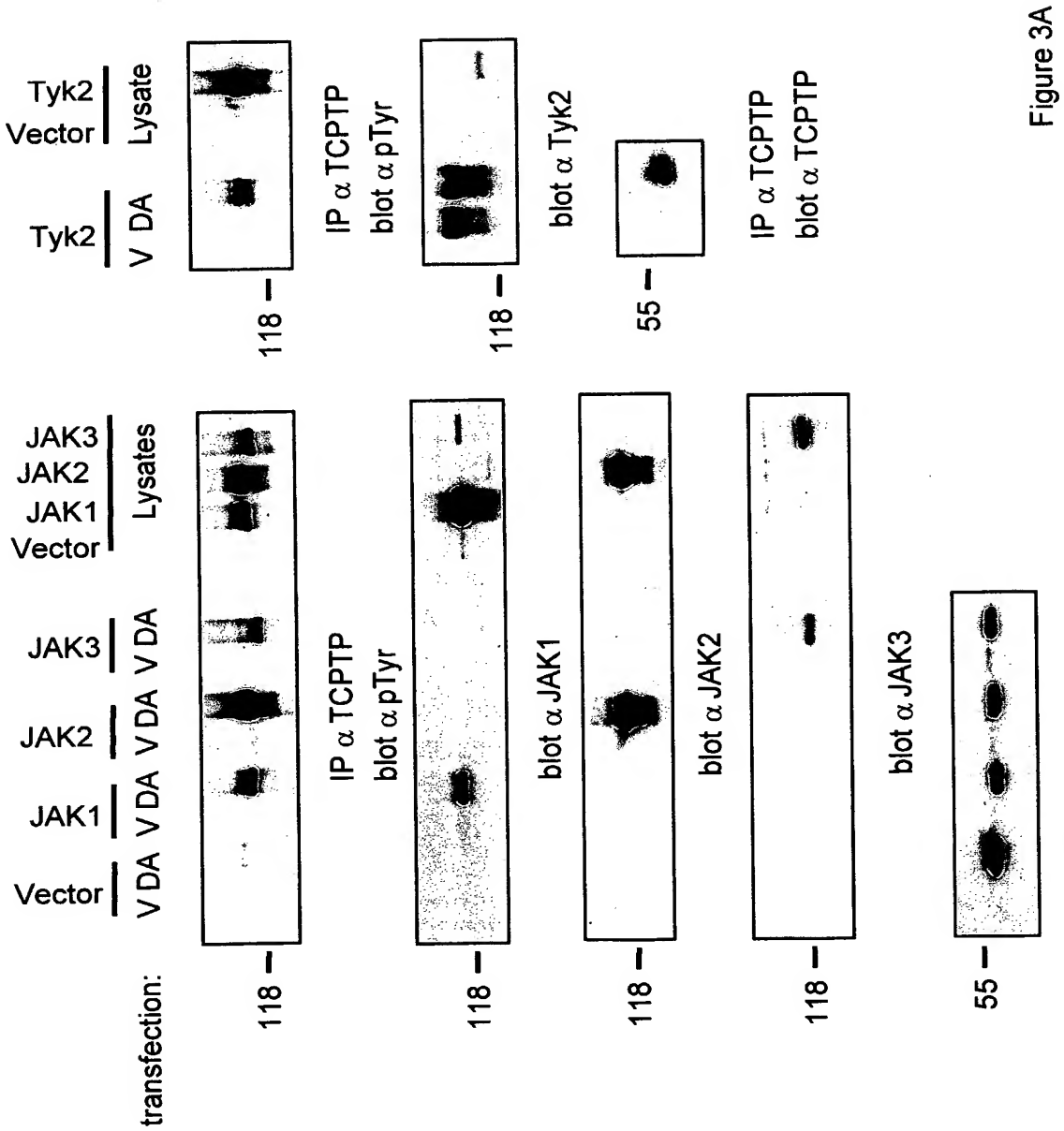
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Figure 3A

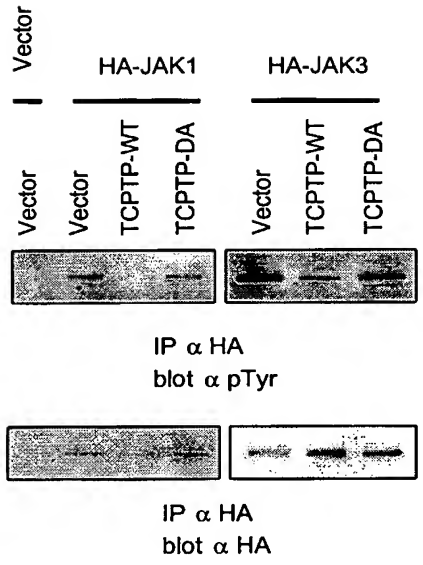
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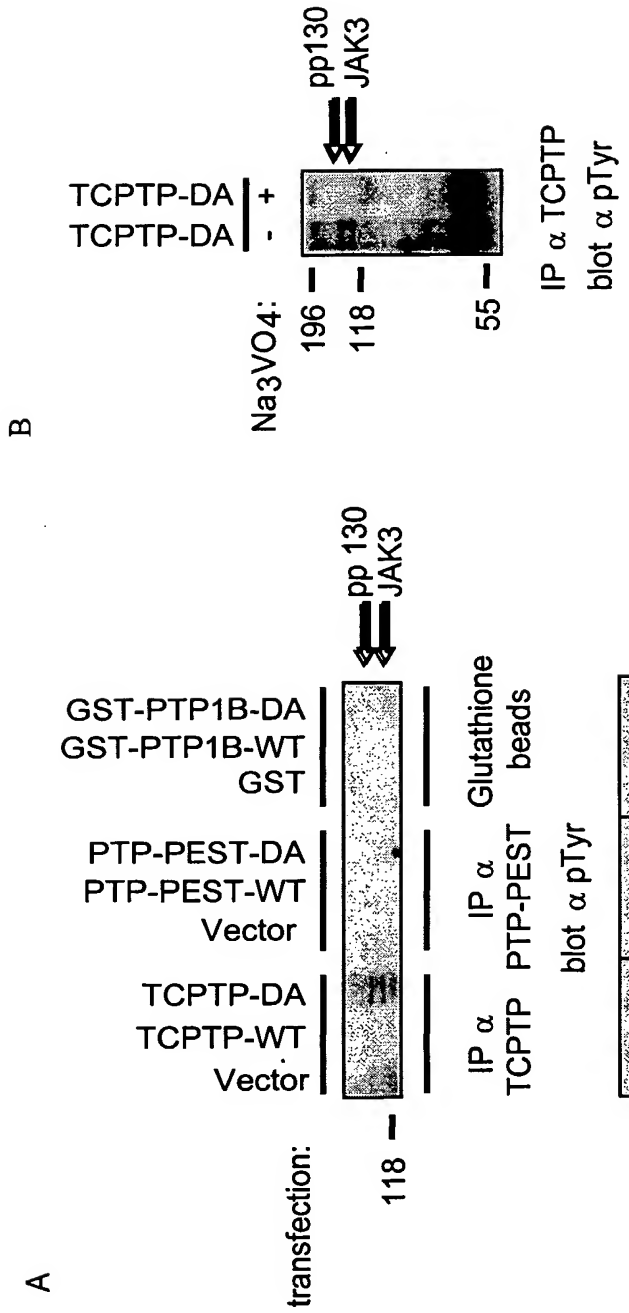
Figure 3B



Figures 4A and 4B

Figures 4C and 4D

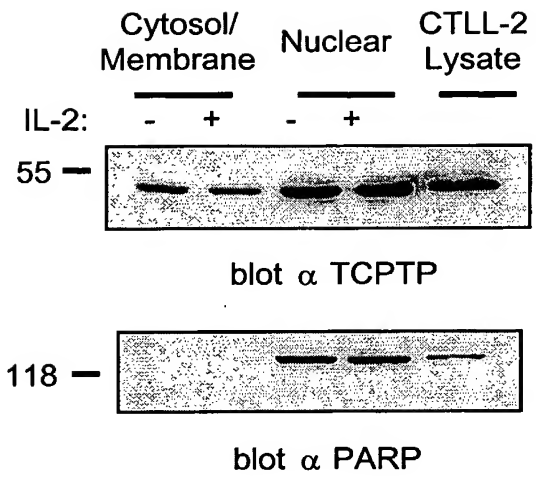

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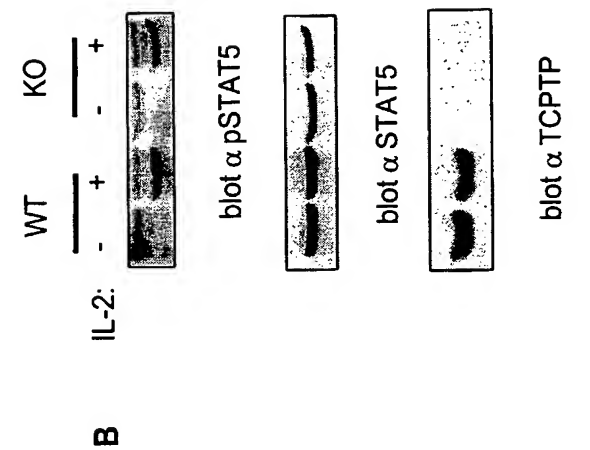
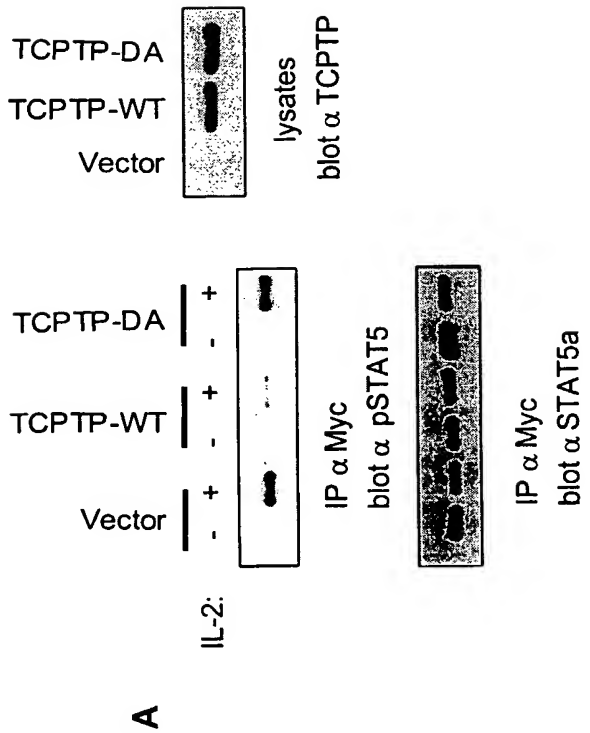
Figure 5





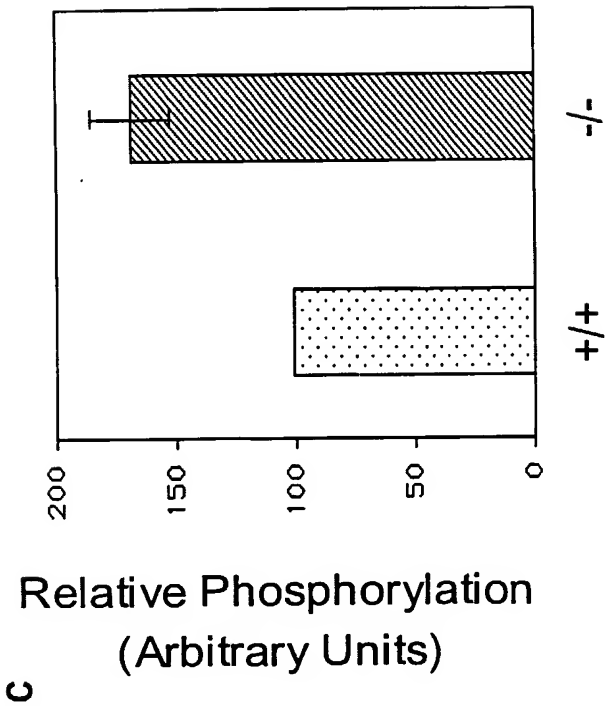


Figure 6a, 6B and 6C

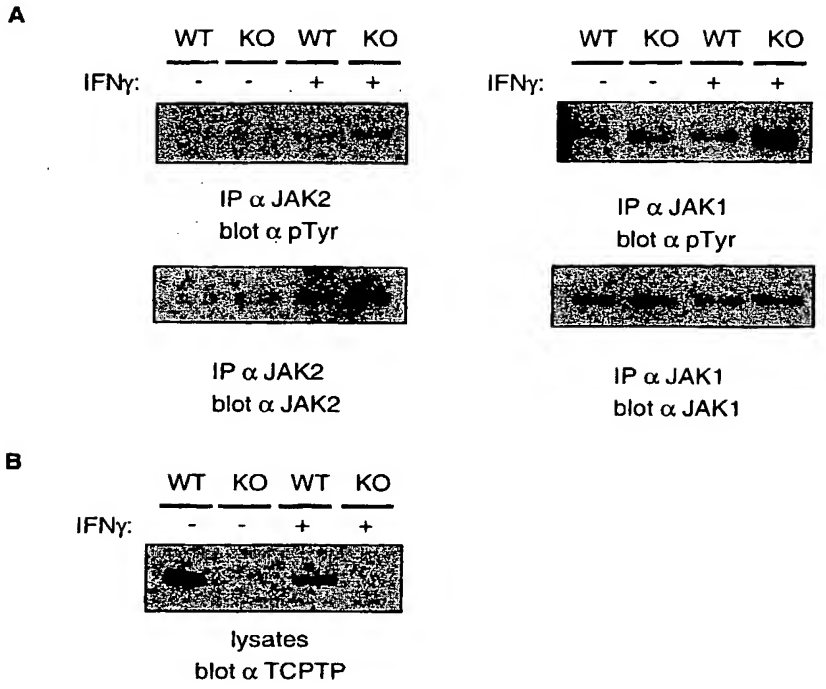

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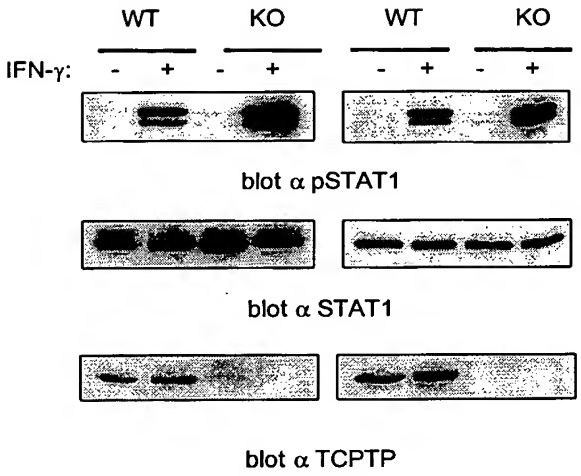
Figure 7

A



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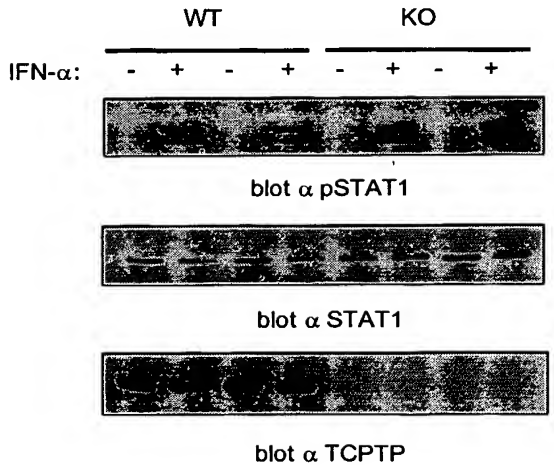

Figures 8A and 8B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 01/01679

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/42 G01N33/566 G01N33/68 A61K38/47 A61P37/04
A61P37/06 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IBARRA-SANCHEZ M. J. ET AL.: "The T-cell protein tyrosine phosphatase" SEMIN. IMMUNOL., vol. 12, no. 4, August 2000 (2000-08), pages 379-386, XP002192148	1-5
Y	the whole document	33,34

X	WO 00 36111 A (IBARRA SANCHEZ MARIA DE JESUS ; UNIV MCGILL (CA); TREMBLAY MICHEL) 22 June 2000 (2000-06-22)	26,27
Y	the whole document	1-5,33, 34

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 March 2002	26/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Marinoni, J-C

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	YOU-TEN KONG E ET AL: "Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 186, no. 5, 1997, pages 683-693, XP002192149 ISSN: 0022-1007 cited in the application	1-5, 32-34
A	the whole document	

Y	WO 99 36548 A (HSC RES DEV LP ;ROIFMAN CHAIM M (CA)) 22 July 1999 (1999-07-22)	32-34
A	the whole document	

Y	FLINT ANDREW J ET AL: "Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 5, 1997, pages 1680-1685, XP002051429 1997 ISSN: 0027-8424 the whole document	32-34

Y	WIMMER MONIKA ET AL: "A non-receptor-type protein phosphotyrosine phosphatase is enriched in secretory vesicles of glucagon - and pancreatic polypeptide - secreting cells of the endocrine pancreas." HISTOCHEMISTRY AND CELL BIOLOGY, vol. 111, no. 2, February 1999 (1999-02), pages 135-142, XP002192207 ISSN: 0948-6143 the whole document	32

A	WO 99 23493 A (UNIV ROCKEFELLER) 14 May 1999 (1999-05-14)	

A	WO 98 27092 A (SUGEN INC) 25 June 1998 (1998-06-25)	

A	MAEGAWA HIROSHI ET AL: "Expression of a dominant negative SHP-2 in transgenic mice induces insulin resistance." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 42, 15 October 1999 (1999-10-15), pages 30236-30243, XP002192535 ISSN: 0021-9258	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MAEGAWA HIROSHI ET AL: "SHPTP2 serves adapter protein linking between Janus kinase 2 and insulin receptor substrates." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 228, no. 1, 1996, pages 122-127, XP002192152 ISSN: 0006-291X	
A	IHLE J.: "The Janus protein tyrosine kinases in hematopoietic cytokine signaling" SEMIN. IMMUNOLOGY, vol. 7, 1995, pages 247-254, XP002192153 page 251, left-hand column, line 11 - line 41	
A	GJORLOFF-WINGREN ANETTE ET AL: "Subcellular localization of intracellular protein tyrosine phosphatases in T cells." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 30, no. 8, August 2000 (2000-08), pages 2412-2421, XP002192154 ISSN: 0014-2980	
A	TIGANIS TONY ET AL: "Epidermal growth factor receptor and the adaptor protein p52Shc are specific substrates of T-cell protein tyrosine phosphatase." MOLECULAR AND CELLULAR BIOLOGY, vol. 18, no. 3, March 1998 (1998-03), pages 1622-1634, XP002192155 ISSN: 0270-7306 cited in the application	
A	TIGANIS TONY ET AL: "Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 34, 1997, pages 21548-21557, XP002192156 ISSN: 0021-9258 cited in the application	
P,X	WO 01 17516 A (NOVO NORDISK AS ;ONTOGEN CORP (US)) 15 March 2001 (2001-03-15) the whole document	1-34
P,X	WO 00 75339 A (ZHANG SHAO HUI ;COLD SPRING HARBOR LAB (US); TONKS NICHOLAS K (US)) 14 December 2000 (2000-12-14) the whole document	1-34

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 6-8 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 11 and 32 are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 6-8, 11, 32

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

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Continuation of Box I.2

Claims Nos.: 1-5, 9-12, 26-28, 33 and 34 partially; 13-25 and 29 completely

Present claims 13-25 and 29 relate to methods defined by reference to a desirable characteristic or property, namely that they involve the provision of an analog of TCPTP.
The claims cover all methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for no such analogs and nothing in the application as originally filed could allow the skilled person to define such an analog.
Additionally, claims 26-28 are directed to an immunosuppressor that blocks the activation of JAK kinase by TCPTP. The claims cover all coumpounds having this property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for no such immunosuppressor except for TCPTP antisense nucleic acids.
In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). This lack of clarity is such as to render a meaningful search over the whole of the claimed scope impossible.
Consequently, the search has not been carried out at all for said claims 13-25, 28 and 29 and only partially for claims 26 and 27.

The same objection applies mutatis mutandis yet partially to claims 1-5, 9-12, 33 and 34 which refer to the possible use of analogs, mimetics or

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

functional derivatives, for which no disclosure is to be found in the application as originally filed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5

   A method for regulating an immune response comprising increasing the phosphatase activity of TC-PTP.

2. Claims: 6-31

   A method of diagnosing an immune disorder by detecting alterations of a TC-PTP/JAK interaction, methods to isolate coumpounds which modulate said interaction, said compounds, etc...

3. Claim : 32

   A method for providing a host with resistance to diabetes by decreasing the level of TCPTP in said host.

4. Claims: 33,34

   The use of TCPTP in the preparation of a medicament for the treatment of an immunological condition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/CA 01/01679

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